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(54) Title: METHODS AND APPARATUS FOR ICE-MEDICATED INTRODUCTION OF SUBSTANCES INTO BIOLOGICAL MATERIAL IN A NON-LETHAL MANNER

(57) Abstract

According to the present invention, a desired substance to be introduced into a cell is added to a desired liquid capable of dissolving, suspending or encapsulating the substance. For the purposes of the specification and claims, solution will be defined to include liquids in which the desired substance is either dissolved or suspended or in part both. The solution is then used to make ice particles that contain the desired substance. These ice particles are then accelerated toward a target tissue whereby at least some of the particles impact upon and enter at least some cells in the target tissue without killing the cells. Once inside the cell, these ice particles then melt, leaving behind the desired substance in the protoplasm of the bombarded cell.

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METHODS AND APPARATUS FOR ICE-MEDICATED INTRODUCTION OF SUBSTANCES INTO BIOLOGICAL MATERIAL IN A NON-LETHAL MANNER

FIELD OF THE INVENTION

This invention relates generally to the non-lethal introduction of foreign substances into the interiors of living cells, and more specifically to the transformation of living cells by the introduction of foreign genes.

BACKGROUND OF THE INVENTION

A variety of techniques are known for the introduction of foreign substances, particularly DNA, into the interiors of living cells without killing those same cells. One class of techniques involves manipulating the cell membrane to make it permeable to DNA molecules. For instance, in bacteria, yeasts and protoplasts of higher plant cells, treatments with chemicals or heat can be used to make the cell membranes "leaky," thereby permitting a desired gene or genes (often integrated in small loops of DNA called plasmids) to be taken up into the cell.

In another class of techniques, DNA is physically injected into living cells, particularly ova, of various animal species. DNA can also be taken up by electrically stimulated cells in a process known as electroporation. The cell is made permeable using a precision laser to burn holes into the cell membrane..

Yet another type of gene introduction can be accomplished using naturally occurring processes. Gene transfer is accomplished by infecting certain susceptible dicotyledonous plant species with a particular bacterial species of the genus Agrobacterium. The bacterium possesses plasmids as a part of its normal complement of DNA. Upon

infection, the bacterium is capable of transferring portions of these plasmids to the cells of the plant it infects. Thus, it is possible to accomplish the introduction of specifically desired genes by engineering Agrobacterium species with recombinant plasmids possessing the desired gene and subsequently infecting a susceptible plant with the engineered bacterium.

Most recently, another type of gene introduction method has been described. Termed "microprojectile bombardment" or "biolistics," this technique involves coating tiny metal spheres with desired DNA, and then accelerating these projectiles into tissues. Acceleration is generally achieved by shooting the microprojectiles from a gun aimed at the target tissue. Once inside the cell, the foreign DNA detaches from the metal sphere and, in certain of the bombarded cells, is incorporated into the host cell's DNA. See, e.g., Sanford, J.C., "The biolistic process," Trends in Biotechnology, 6:299-302 (1988); Klein, T. et al. "Stable genetic transformation of intact Nicotiana cells by the particle bombardment process," Proc. Nat. Acad. Sci. USA 85:8502-8505 (1988); E.P. Application, S.N. 87310612.4; E.P. Application, S.N. 88306613.6; and E.P. Application, S.N. 88402481.1.

Unfortunately, each of the above methods has distinct disadvantages. The methods may be taxonomically limited, such as with Agrobacterium-based methods. Membrane permeability methods have limited application with organisms presenting cell walls. Electroporation methods achieve only a very low efficiency of stable transformation. Several methods, such as microinjection and laser-mediated cell membrane manipulation are extremely tedious,

time consuming and require highly specialized equipment.

Although the biolistics approach avoids some of these drawbacks, the design and composition of the microprojectiles presents disadvantages unique to the method. For instance, metal microprojectiles can easily clump into larger aggregates that, upon impact, can cause significant cell damage. The type of substances that can be introduced into the cell is limited to negatively charged molecules such as DNA and RNA. The amount of DNA or RNA carried with each microprojectile cannot be easily controlled or measured. The long-term chemical and physical effects of the metal microprojectiles inside the cell are unknown. Finally, the use of metallic agents requires considerable preparation time, thus affecting the economic efficiency of the method.

SUMMARY OF THE INVENTION

The foregoing and other problems associated with known methods of introducing substances into cells are overcome by the present invention. According to the present invention, a desired substance to be introduced into a cell is added to a desired liquid capable of dissolving, suspending or encapsulating the substance. For the purposes of the specification and claims, solution will be defined to include liquids in which the desired substance is either dissolved or suspended or in part both. The solution is then used to make ice particles that contain the desired substance. These ice particles are then accelerated toward a target tissue whereby at least some of the particles impact upon and enter at least some cells in the target tissue without killing the cells. Once inside the cell, these ice particles

then melt, leaving behind the desired substance in the protoplasm of the bombarded cell.

The method of the present invention is superior to existing methods in that it provides a way to introduce not only DNA and RNA, but also non-negatively charged substances, including but not limited to proteins, enzymes, protein/DNA complexes, bacteria, viruses, hormones, etc. into living cells. The ability to introduce enzymes and proteins simultaneously with DNA and RNA may materially improve DNA/RNA viability rates as well as transformation rates of individual cells. Further, the ice projectile ceases to exist after it melts, thereby leaving no residue that may poison or otherwise contaminate the bombarded cell at some future time. Because the ice projectiles are produced from a solution of known concentrations, it is easy to control and predict the amount of DNA or other substance to be delivered to a particular cell. Also, ice particles may be less likely to clump and thus less likely to cause fatal trauma to bombarded cells. Further, it is possible, for a particle of a given diameter, to incorporate more of the desired substance than can be incorporated onto the surface of a metal particle.

It is therefore an object of the present invention to introduce a variety of substances into biological material in a non-lethal manner.

It is another object of the present invention to accomplish substance introduction with a minimal amount of trauma to the bombarded cell.

It is a further object of the present invention to accomplish substance introduction without producing a by-product that may in time deleteriously affect the functioning of the bombarded cell.

It is yet another object to provide a method of introducing foreign substances into cells that is both efficient and cost-effective.

A further object of the present invention is to provide a method of substance introduction that is efficacious for a wide variety of different species and tissue types.

It is still another object of the present invention to provide a method of introducing DNA into cells in which the rate of transformation is high.

Another object of the present invention is to provide a simple and cost effective method for generating ice particles of an appropriate size range containing the desired substance.

Still another object of the present invention is to provide an apparatus for the production of ice particles.

A further object of the present invention is to provide a simple, self-contained apparatus for accelerating ice particles toward a target tissue that is inexpensive to construct and easy to operate.

These and other objects of the present invention will be more readily understood upon consideration of the following detailed description of embodiments of the invention and the drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a view in cross-section of the ice particle generator of the present invention.

FIG. 1B is a view in cross-section of an apical portion of the ice particle generator of the present invention.

FIG. 2A is a cutaway view in perspective of the pellet of the present invention.

FIG. 2B is an exploded cutaway view in perspective of the pellet of the present invention.

FIG. 3A is a view in cross-section of the ice particle accelerator of the present invention.

FIG. 3B is a view in cross-section of the vacuum chamber portion of the ice particle accelerator of the present invention.

FIG. 3C is a schematic view in perspective of the support apparatus within the vacuum chamber of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Referring to FIG. 1A, the ice particle generator **10** of the present invention will now be described. The generator **10** is comprised of an inner shell **12** which defines a freezing chamber **14**. The freezing chamber **14** is provided with a gas feed line **20** from a regulated nitrogen tank. It is also provided with a nitrogen gas recycling line **16** which leads from coolant chamber **36**.

The freezing chamber **14** is further provided with a nebulizer **22** for dispersing the solution into a mist of very fine particles and a mist feed line **24** permitting the mist to enter the freezing chamber **14**. The nebulizer **22** is attached externally to a gas feed line **23** that is attached a tank of compressed nitrogen or other substantially inert gas. The nebulizer should be capable of forming mist particles that upon freezing are of an average diameter of 1 micron. One such suitable nebulizer is produced by De Vibliss Company, model no. 644.

The freezing chamber **14** is closed above by emplacement of a stopper **26** at the apex and by an end segment **27** at the base of the inner shell **12**. The stopper **26** is provided with a thermometer **28** for gauging the temperature of the freezing chamber, and is provided with a vent hole **29**. Placed upon the end segment **27**, at the base of the freezing chamber,

there is provided a pellet holder **32** in which are emplaced pellets **50**. Above the pellet holder **32** is placed a funnel **30**, supported by a three-legged ring stand **31**, for collecting the ice particles formed from the mist produced by nebulizer **22** and for directing the particles into the pellets **50**.

The generator **10** further comprises an outer shell **34** positioned to surround the inner shell **12** for substantially much of its length. The position of the outer shell **34** with respect to the inner shell **12** defines an coolant chamber **36**. The coolant chamber **36** is provided with a coolant feed **38** and a coolant drain **40**, which is provided with a flow valve **42**.

The inner and outer shells **12** and **34** sit atop a support **44**. End segment **27** is supported by a vertically adjustable tripod **48** which allows the insertion, height adjustment, and removal of end segment **27**, pellet holder **32**, three-legged ring stand **31**, funnel **30** and pellets **50**.

The inner and outer shells **12** and **34** may be composed of any suitable material resistant to cracking or other degradation that may result from rapid temperature changes. Suitable materials include galvanized steel, stainless steel, aluminum, sheet metal, copper and other materials such as ceramics and plastics. Metals are preferred because they are inexpensive and easy to form. The stopper **26** is preferably composed of rubber. The collection funnel **30** is preferably composed of some smooth-surface polymer plastic or glass resistant to rapid temperature changes. The pellet holder **32** is preferably composed of a foamed rubber or polymer plastic.

A suitable air rifle pellet is produced by Prometheus Company. Pellet **50** is comprised of two

parts, a core **56** comprised of aluminum having a broad rounded base which has been filed flat and a narrower projecting columnar portion, and a skirt **54** comprised of plastic that is configured to be received over the columnar portion of core **56**. The pointed or curved base of core **56** has been filed flat in order to permit the casing **52** to be loaded and fired from gun **110** in a position that is backwards relative to its intended orientation.

Alternatively, the skirt **54**, may be removed from the core and sealed on one end. Sealing may be accomplished by any appropriate method such as taping, gluing, heat sealing, etc.

Production of ice particles using the generator **10** described above is accomplished as follows. First, the desired substance to be introduced into the target cell must be selected. In the case of many substances such as proteins, enzymes, etc., all that is required is the dilution of the substance in an appropriate, liquid to a desired concentration. Water is preferred. Where transformation of the cell is desired, an appropriate vector capable of cell transformation is selected. The vector is then manipulated to include the desired gene or genes to be integrated into the genome of the target cells. Once manipulation is complete, the vector can be multiplied and isolated by various techniques that are well known in the art and then dissolved and diluted in a solution to a desired concentration. This solution is then maintained under conditions sufficient to ensure that the biological properties of the selected substance are maintained.

Prior to ice particle generation, the generator **10** is conditioned as follows. Pellets **50** are placed securely in the pellet holder **32**. The pellet holder **32** is then placed beneath the collection funnel **30**

(which is supported by the three-legged ring stand **31**) atop end segment **27**. The end segment **27** is then raised into the bottom of the freezing chamber **14** by means of tripod **48**. Stopper **26** with thermometer **28** should be securely inserted into the upper end of freezing chamber **14**.

Once the generator **10** is assembled, the production of ice particles may begin. The freezing chamber **14** is flushed with compressed nitrogen via feed gas line **20**. It is preferred to feed the nitrogen into the freezing chamber **14** at about 5 psi for a period of two minutes. Flushing with nitrogen gas ensures that all water vapor has been evacuated from freezing chamber **14** via vent **29**. Failure to rid chamber **14** of water vapor may result in the formation of foreign ice particles that can contaminate the desired ice particles later to be generated.

Following chamber flushing, coolant is added to the coolant chamber **36** through coolant feed **38** in an amount sufficient to fill the chamber. This will cause the temperature within the freezing chamber to drop. The preferred coolant is liquid nitrogen. A sufficient amount of cooling has taken place when the thermometer **28** records a temperature of 5°C below the freezing point at the desired length. Cooling efficiency is increased when gaseous nitrogen that boils off coolant chamber **36** is transferred into the bottom of freezing chamber **14** via gas recycling line **16**.

Once the freezing chamber **14** has reached the proper temperature, the nebulizer **22** is filled with the sample solution prepared previously. In the De Vibliss nebulizer disclosed above, it is preferred to add approximately 5-7 ml of the desired solution. At this time, nitrogen gas is fed into nebulizer **22** through feed **23** at about 5 psi. This begins mist

formation, with the mist entering the freezing chamber **36** via mist feed line **24**.

As the mist particles fall through freezing chamber **14**, they freeze and are collected in collection funnel **30**, which directs the particles into the pellets **50**. Ice particles collect in pellets **50**, largely under the influence of gravity. Material comprised solely of such free frozen granules is referred to as "powder." A certain proportion of the ice particles generated will collect on the sides of funnel **30**. These may be scraped and packed manually. Frozen material collected in this fashion is referred to as "packed."

Generally speaking, nebulization should be carefully regulated to ensure that the biological properties of the desired substance in solution are preserved. Where transformation vectors are employed, excessive pressure or nebulization time can result in shearing of the DNA strand, rendering the vector incapable of transforming the target cell. Thus, the pressure and length of time used to nebulize a solution containing such vectors will vary according to the fragility of the vector. Such parameters can easily be determined experimentally using techniques well known in the art, whereby fragility of the vector is generally proportional to vector size and configuration.

As an alternative to ice particle generation through nebulization, the solution containing the desired substance may be "block-frozen." The ice blocks can then be chipped or ground up and manually packed into an appropriate pellet casing.

As an alternative to nebulized particle generation, pellets containing block frozen ice can be fired intact with the particles being generated upon impact with the stopping plate **138**. As a

further alternative, the solution can be block frozen in the pellet and then subjected to sonication which will generate fracture lines within the ice block, thereby causing a more efficient shattering upon impact.

Care should be taken to ensure that the sample, once frozen, the sample does not thaw before it is used. Care should be taken to ensure that the pellets **50** containing packed block-frozen solution or ice particles are kept frozen until used. One suitable method is to pack the pellets into a suitable container, such as a block of foam plastic polymer with receptacles for receiving the pellets. The plastic block with pellets **50** can then be stored under cold conditions, such as being floated on liquid nitrogen in a covered, insulated container.

Turning now to FIGS. 3A and 3B, the ice particle accelerator **100** will be described. Accelerator **100** is comprised of three principal parts: a gun **110**, a housing **120**, an a support structure **130**.

Gun **110** provides the accelerating force. the make and specifications of gun **110** will depend upon the type of pellet used. However, acceleration using chemical charges or other heat intensive methods are not compatible with ice particle acceleration in the present invention. In the methods of the present invention it is preferred to use pellets designed to be shot from air guns. Smooth bored guns are preferable over rifles in that frictional heat upon discharge is reduced resulting in a greater amount of the ice remaining frozen.

Housing **120** is configured to fit over support structure **130** to form a tight seal so that a partial vacuum can be created and maintained interior to housing **120**. Housing **120** is provided with vacuum hose adapter **122** and vacuum gauge **124**. The vacuum

gauge **124** is provided with a three way valve **125** through which air may be reintroduced into the interior housing **120** when a vacuum is no longer required. The housing can be comprised of any convenient material that is sufficiently strong to contain a partial vacuum without structural failure. Plexiglass is preferred because it is inexpensive and easy to form.

Support structure **130** is designed to receive, position and hold the tissue sample for impact during acceleration of the ice particles. The support structure comprises a base plate **132** and a sandwich comprised of a seal **134** above and a plexiglass sheet **135** which is placed below. Base plate **132**, seal **134** and sheet **135** are provided with an aperture **146** extending continuously through the two plates when joined. Aperture **146** provides an entry point into the accelerator for gun **110** which is positioned below. Base plate **132** is further provided with a bore hole **147** through which a set screw can be inserted and used to position and secure the barrel of gun **110** in proper alignment within aperture **146**. The base plate **132** should be durable and strong and is comprised of metal, preferably steel.

Four rods **136** are secured to the base plate **132**. The interior structure is stabilized by the addition of a structural support plate **144** which is secured to rods **136** opposite the base plate **132**. The rods are threaded along their length to accommodate bolts **137** that are used to position various elements of support structure **130** that will presently be described. Just above the base plate **132** is positioned a stopping plate **138**. Stopping plate **138** has a hole located at each of the four corners of the plate to allow stopping plate **138** to slide down and over the ends of rods **136**. Stopping plate **138** is positioned at a

desired distance from seal **134** by resting the plate on bolts **137** that have been screwed down along rods **136** to a desired position. Once in place, stopping plate **138** is secured into place by a second set of bolts **137**.

Stopping plate **138** is provided with an aperture **148** positioned on the plate so that aperture **148** is in direct vertical alignment with aperture **146**. After discharge, the pellet containing the ice particles will impact at aperture **148**. Aperture **148** should be small enough in diameter to stop the pellet **50** but large enough to permit the ice particles to pass and continue toward the target tissue. Stopping plate **138** is comprised of any durable material capable of sustaining repeated bullet impacts without rapid deterioration; steel is preferred.

Above stopping plate **138** is positioned a metal deflector **140**. The tip of deflector **140** should be positioned over aperture **148** of stopping plate **138** so that discharged ice particles that have passed through aperture **148** go on to strike deflector **140** thereby being dispersed into smaller fragments and over a larger area. This helps to minimize the incidence of large frozen fragments that might lethally damage tissue on impact and helps to ensure that a greater area of the tissue will be impacted by the ice particles. Deflector **140** is laterally attached to one the rods **136** and may be positioned at any desired point along a rod in the same way as stopping plate **138**. The deflector is a length of sterilizable material (steel is preferred) that can withstand repeated impact. It has a conical tip extended to a sharp point which further aids in dispersing ice projectiles. Use of a deflector is not required in all instances.

Above deflector **140** are positioned support wires **142**. Bridging the support wires are a pair of adjustable wires **143**. The adjustable wires **143** are slidable along support wires **142** so as to accommodate target material of varying size and shape. Target tissue is placed on the adjustable wires **143** and backing plate **144**. It should be understood that the positions of each element of the infrastructure of support structure **130** can be adjusted relatively to one another along rods **136**.

Having described the ice particle accelerator **100**, the method of introducing foreign substances according to the present invention will now be described. Stopping plate **138** is secured to rods **136** at a distance from gun **110** sufficient to allow an optimum number of ice particles to be accelerated through aperture **148**. Deflector **140**, if desired, may then be positioned above stopping plate **138** at a sufficient distance to ensure that ice particles are scattered for impact over a large area of the target tissue. The desired target tissue is placed on adjustable wires **143** at a desired distance from gun **110** to ensure that the tissue is impacted with ice particles in a substantially non-lethal manner.

Once the supporting structure with target tissue is set up, outer housing **120** is placed over support structure **130** such that a good seal is formed between the support structure **130** and the outer housing **120**. A moderate vacuum is then applied to the chamber to reduce heat of friction between the ice particles and the atmosphere. The amount of vacuum varies according to tissue type, although it is generally preferred to reduce atmospheric pressure to between 20 to 30 inches of mercury prior to acceleration. At this time, the temperature of the accelerator should

be lowered sufficiently to ensure that the ice particles remain frozen prior to tissue impact.

A pellet **50** containing nebulized or block frozen solution containing the desired substance is retrieved from storage and loaded into the gun **110** so that the open end of pellet **50** which contains the core **56** rests at the bottom of the gun chamber. This ensures that the open end of pellet **50** which contains the frozen solution will be facing toward aperture **148**. After loading, the gun is discharged, thereby accelerating pellet **50** toward stopping plate **138**. Pellet **50** is stopped upon impact with stopping plate **138**, but the frozen solution within pellet 50 continues through aperture **148**. As a general matter, impact with the stopping plate fragments the frozen solution, if block frozen, or mostly disperses the frozen solution, if nebulized, into ice particles as the pellet strikes the stopping plate.

Having passed through aperture **148**, the ice particles are further dispersed as they strike deflector **140**. The deflector acts to enlarge the impact zone of the ice particles on the target tissue by making the trajectories of the ice particles more oblique relative to their original paths. After passing the deflector, the ice particles travel on to and imbed in the target material. After impact, the target material may be left in place and reimpacted as desired. Once the desired number of impacts has been achieved, air is bled into the housing **120** via the three way valve **125** until pressure inside the accelerator **100** is equal to atmospheric pressure. The housing is then separated from the rubber seal **134** and the target material removed.

EXPERIMENTAL

The use of methods and apparatus of the above described invention in transforming plant tissues will now be described.

Plasmid pBI121 (obtained from CLONTECH Laboratories, Palo Alto, California) was selected for the gene vector because it contains genes for the enzymes beta-glucuronidase (GUS) and neomycin phosphotransferase II (NPT II). When expressed in an organism, GUS can form an insoluble blue dye in the presence of the reagent 5-Bromo-4-Chloro-3-indolyl beta D-glucuronic acid, "X-gluc." NPT II produces an enzyme that inactivates the antibiotic kanamycin which otherwise kills normal plant cells. These genes are effectively "markers" in that their expression indicates that the cell has taken up the plasmid and the genetic information of the plasmid is being expressed. Of course, further engineering of the plasmid to include a specific desired foreign gene will be required in application of the invention described herein.

Replication of the plasmid to produce a sufficient quantity of plasmids for experimentation was accomplished by transforming competent cells of Escherichia coli strain JM109 (obtained form Promega, Inc.) using standard, well known methods. The transformed E. coli were grown to a high density in a rich liquid medium of a standard composition well known in the art for effective E. coli culture.

Once a sufficient volume of culture was obtained, the pBI121 plasmids were isolated from the E. coli cells by alkaline lysis, followed by a cesium chloride density gradient centrifugation according to procedures well known in the art. The plasmids were precipitated by ethanol and dried according to standard techniques in the art. The plasmids were

then redissolved in a sterile 10 mM aqueous solution of sodium chloride to a concentration of 0.1 mg/ml. Standard Tris-EDTA buffer was not used to redissolve the plasmids in order to avoid the introduction of potentially toxic compounds into the target tissue.

The plasmid containing solution was then frozen according to the techniques set forth and described above. In instances where the ice particles are formed according to the nebulization technique of the present invention, care was taken to avoid treatment that would destroy the integrity of the plasmid and thus its ability to achieve transformation of the target tissue. Thus, it was found that nebulization pressures in excess of 5 psi and nebulization times in excess of ten minutes should be avoided when using plasmid pBI121 as the desired gene vector. Pressures and times in excess of these figures will shear substantially all the plasmid into segments too small to effect transformation.

The prepared pellets were loaded then accelerated toward the target material positioned within the ice particle accelerator according to the method described above. After this treatment, the material was incubated in a sterile container at 25°C under a relative humidity approaching 100% to permit transformation and expression.

After incubation, the treated tissues were histochemically stained for GUS activity using "X-gluc" substrate according to the methods set forth in the manufacturer's directions. The GUS enzyme acts on the "X-gluc" substrate, resulting in an insoluble blue dye that forms crystalline deposits in transformed cells. The target tissue was allowed to incubate overnight at 37°C in the "X-gluc" substrate, and was examined thereafter to ascertain the number of transformed cells.

Example 1a

A comparison of the transformation efficiencies of nebulized frozen plasmids versus block frozen plasmids on corn leaves and tobacco leaves was conducted using the methods described above. The results are presented in Table I.

Table I

Transformation of Tissue Using Block Frozen vs. Nebulized Plasmid (pBI121)

	Transformants per shot (average)	
Tissue	Block Frozen(a)	Nebulized(b)
Corn Leaf	2.5	3.7
Tobacco Leaf	6.0	0.5

a) 10 ul. of plasmid (0.1 mg/ml)

b) Pellets filled with nebulized plasmid powder (0.1 mg/ml)

These results show that both menocots and dicots can be transformed by the method of the present invention. Further, the efficiency of block frozen v. nebulized ice projectiles in transformation will depend on species and tissue type.

Example 2

In this example, the effect of varying the volumetric amount of frozen plasmid solution having a standard plasmid concentration of 0.1 mg/ml was investigated. The results are set forth in Table II.

Table II

Transformation of Tissue Using Varying Volumes of Block Frozen Plasmid (pBI121)

Tissue	Volume (μl)	Transformants per shot(average)
Corn leaf	3	2.0
	10	6.3
Tobacco Suspension	20	23.0
cells	50	57.0

From the experiments, it is apparent that different types of tissue can be transformed using the method of the present invention. Further, an increase in the volume of the block frozen plasmid appears to achieve an increase in transformation rate.

It is now apparent that the apparatus and methods of the present invention for introducing substances into cells in a non-lethal manner show marked improvements over known apparatus and methods. It is to be understood that although certain preferred embodiments have been disclosed and described above, other embodiments are possible without departing from that which is the invention described herein. It is intended therefore that the invention be defined by the claims that follow as well as equivalents thereof.

We claim:

1. A method for introducing substances into biological material in a non-lethal manner comprising the steps of:

a) preparing ice particles from a solution of a desired substance;

b) providing biological material as a target; and

c) physically accelerating the ice particles containing the desired substance toward the biological material under conditions sufficient to ensure that at least some of the ice particles lodge in the biological material in a non-lethal manner and melt to release the desired substance.

2. The method according to Claim 1 wherein the solution is water-based.

3. The method according to Claim 1 wherein the ice particles are formed by nebulizing the aqueous-based solution of the desired substance into a fine mist for such time and under conditions sufficient to form ice particles.

4. The method according to Claim 3 wherein the ice particles have diameters of less than 10 microns.

5. The method according to Claim 1 wherein the ice particles are collected into and accelerated within a pellet.

6. The method according to Claim 5 further comprising the step of providing stopping means for halting progress of the pellet without halting progress of the ice particles.

7. The method according to Claim 6 wherein said stopping means comprises a stopping plate.

8. The method according to Claim 5 further comprising the step of providing a dispersion means for scattering the particles prior to impact with the target.

9. The method according to Claim 8 wherein said dispersion means comprises a deflector.

10. A method for introducing substances into biological material in a non-lethal manner comprising the steps of:

a) nebulizing an aqueous-based solution of a desired substance for such time and under conditions sufficient to produce ice particles containing the desired substance;

b) collecting the ice particles into a pellet;

c) providing biological material as a target;

d) positioning stopping means for halting the progress of the pellet between the pellet and the target;

e) positioning dispersion means for scattering the ice particles between the stopping means and the target; and

f) accelerating the pellet toward the target in such a manner that the pellet is stopped by but the ice particles pass through

said stopping means, the ice particles then striking and becoming scattered by said dispersion means, such that at least some of the ice particles lodge in the target in a non-lethal manner and melt to release the desired substance.

11. A method for introducing substances into biological material in a non-lethal manner comprising the steps of:

a) freezing a desired quantity of an aqueous-based solution of a desired substance to form an ice block within a pellet;

b) providing biological material as a target;

c) positioning stopping between the pellet and the target means for halting the progress of the pellet casing and disrupting the integrity of the ice block into ice particles;

d) positioning between the stopping means and the target dispersion means for scattering the ice particles; and

e) accelerating the pellet toward the target in such a manner that the pellet is stopped and the ice block disrupted into ice particles by said stopping means, the ice particles then striking and becoming scattered by said dispersion means, such that at least some of the ice particles lodge in the target in a non-lethal manner and melt to release the desired substance.

12. The method according to Claim 11 wherein the solution is water-based.

13. A method for genetically transforming cells comprising the steps of:

a) preparing ice particles of a solution of copies of a DNA sequence suitable for expression;

b) providing cells as a target;

c) physically accelerating the ice particles containing the DNA sequence toward the cells in such a manner that at least some of the ice particles lodge in a non-lethal manner in the interior of at least some of the cells and melt to release the DNA; and

d) confirming the existence of the DNA in the cells.

14. The method according to Claim 13 wherein the solution is water-based.

15. The method according to Claim 13 wherein the ice particles are formed by nebulizing the aqueous-based solution into a fine mist for such time and under such conditions sufficient to form ice particles.

16. The method according to Claim 15 wherein the ice particles have diameters of less than 10 microns.

17. The method according to Claim 13 wherein the ice particles are collected into and accelerated within a pellet.

18. The method according to Claim 17 further comprising the step of providing stopping means for halting progress of the pellet without halting progress of the ice particles.

19. The method according to Claim 18 wherein the stopping means comprises a stopping plate.

20. The method according to Claim 13 further comprising the step of providing a dispersion means for scattering the particles prior to impact with the target.

21. The method according to Claim 20 wherein said dispersion means comprises a deflector.

22. A method for genetically transforming cells comprising the steps of:

a) nebulizing a water-based solution of copies of a DNA sequence suitable for expression for such time and under conditions sufficient to produce ice particles containing the DNA;

b) collecting the ice particles into a pellet;

c) providing cells as a target;

d) positioning stopping means for halting the progress of the pellet between the pellet and the target;

e) positioning dispersion means for scattering the ice particles between the stopping means and the target; and

f) accelerating the pellet toward the target in such a manner that the pellet is stopped by but the ice particles pass through said stopping means, the ice particles then striking and becoming scattered by said dispersion means, such that at least some of the ice particles lodge in the target in a non-lethal manner and melt to release the DNA; and

g) confirming the existence of the DNA in the cells.

23 A method for genetically transforming cells comprising the steps of:

a) freezing a desired quantity of a solution of copies of a DNA sequence suitable for expression to form an ice block within a pellet casing to form a pellet;

b) providing biological material as a target;

c) positioning stopping between the pellet and the target means for halting the progress of the pellet and disrupting the integrity of the ice block into ice particles;

d) positioning between the stopping means and the target dispersion means for scattering the ice particles; and

e) accelerating the pellet toward the target in such a manner that the pellet is stopped and the ice block disrupted into ice particles by said stopping means, the ice particles then striking and becoming scattered by said dispersion means, such that at least some of the ice particles lodge in the target in a non-lethal manner and melt to release the DNA; and

f) confirming the existence of the DNA in the cells.

24. The method according to Claim 23 wherein the solution is water-based.

25. The method according to Claims 1, 10, 11, 13, 22 or 23 wherein the acceleration of the ice particles is conducted under a partial vacuum.

26. The method according to Claims 1, 10, 11, 13, 22 or 23 wherein the acceleration of the ice particles is conducted under a partial vacuum of from 10 to about 30 inches of mercury.

27. An apparatus for delivery of ice particles containing a desired substance into a target tissue in a non-lethal manner comprising:

a) a housing having a base member and provided with a single continuous interior chamber, said housing provided with vacuum delivery means for creating a vacuum within the chamber when said vacuum delivery means is connected to a source of vacuum;

b) support means for arraying and positioning components of the apparatus, said support means interior to said housing and attached to said base member;

c) accelerating means for imparting an impelling force to the ice particles attached to and passing through said base member of said housing;

d) dispersion means for scattering ice particles within said housing, said dispersion means secured to said support means; and

e) target positioning means for aligning the target within said housing and securing the target for impact by the ice particles, said target positioning means secured to said support means opposite said base member relative to said dispersion means.

28. An apparatus for generating ice particles from a solution of a desired substance:

a) a chamber capable of being sealed in an air-tight fashion;

b) refrigeration means for lowering the temperature within the chamber below the freezing point of the solution;

c) delivery means for introducing a mist of aqueous particles into said inner chamber, said means connected apically to said inner chamber;

d) nebulizing means for generating a mist from an aqueous solution; said nebulizing means connected to said delivery means;

e) means for collecting ice particles after they are formed in said chamber,

wherein mist delivered into said chamber falls through said chamber and freezes into ice particles when said chamber is cooled below the freezing point of the mist.

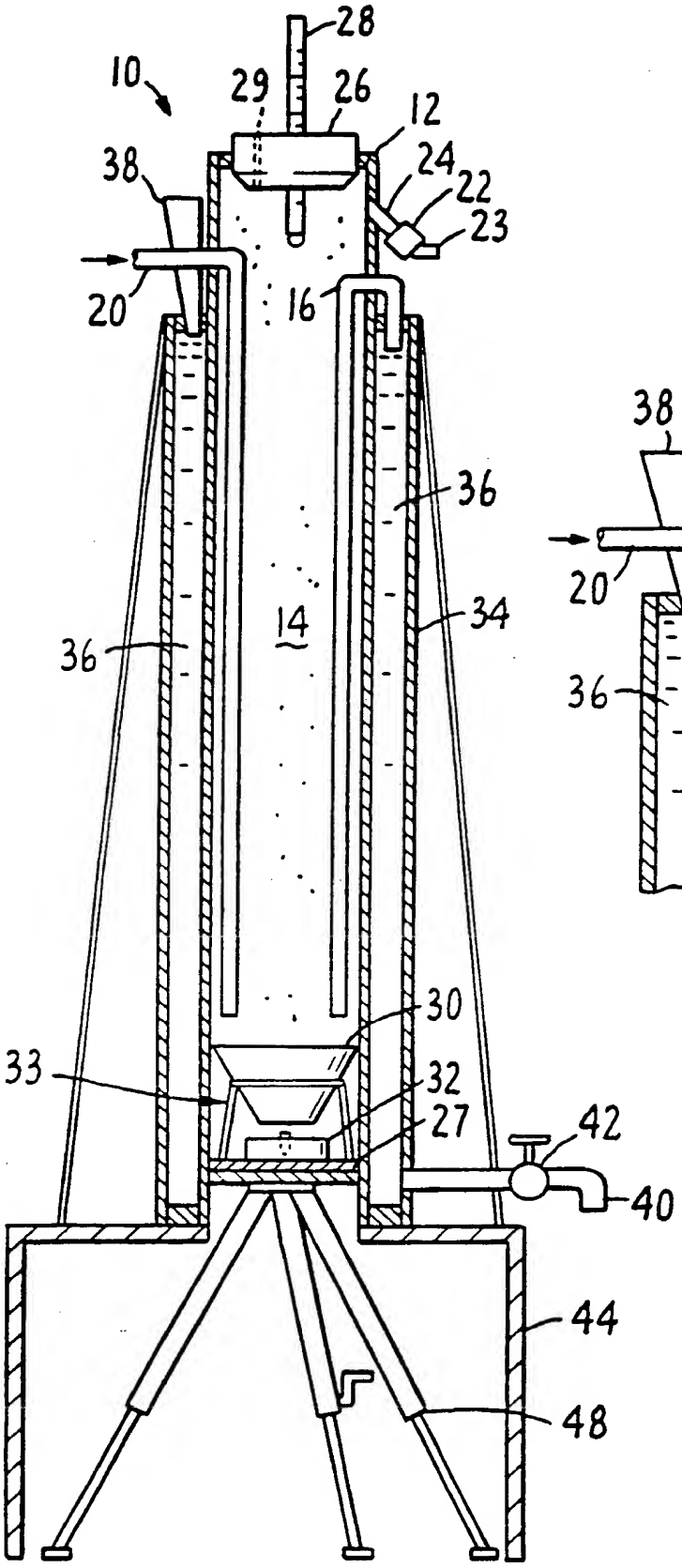

FIG. 1A.

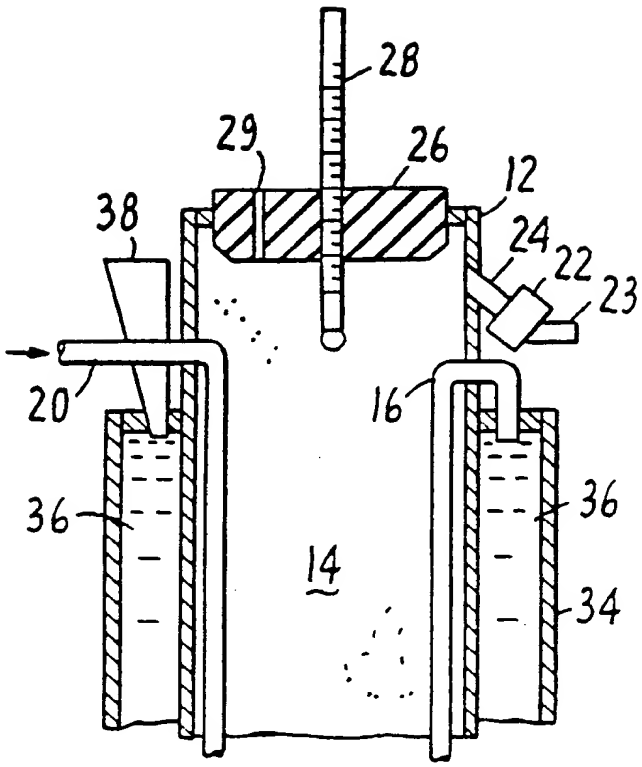

FIG. 1B.

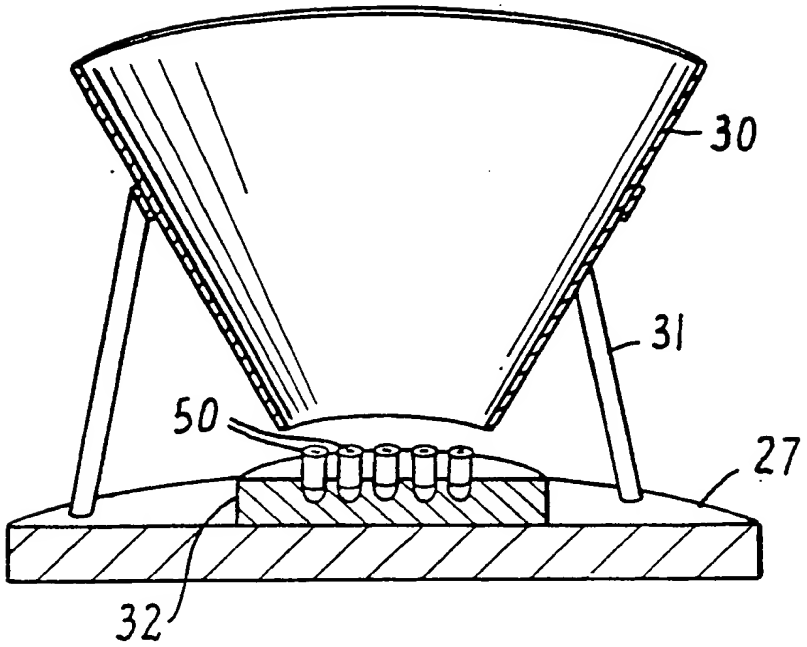

FIG. 1C.

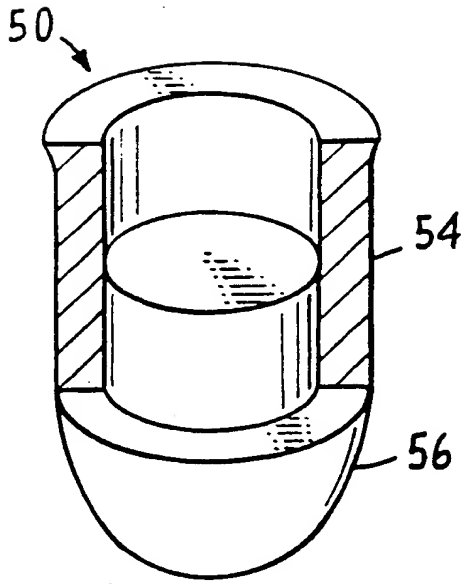

FIG. 2A.

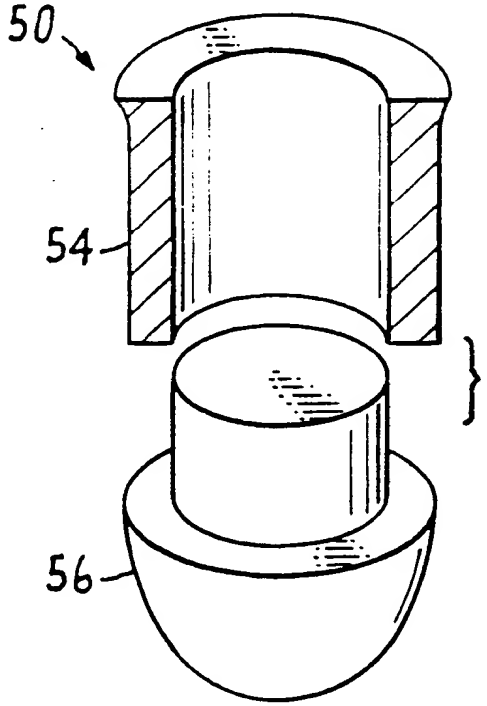

FIG. 2B.

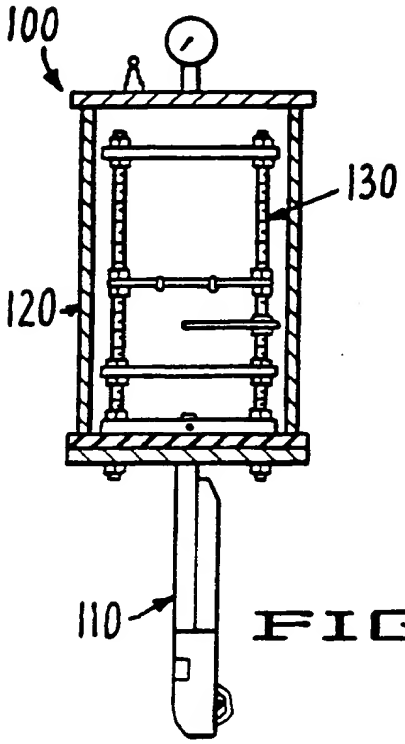

FIG.3A.

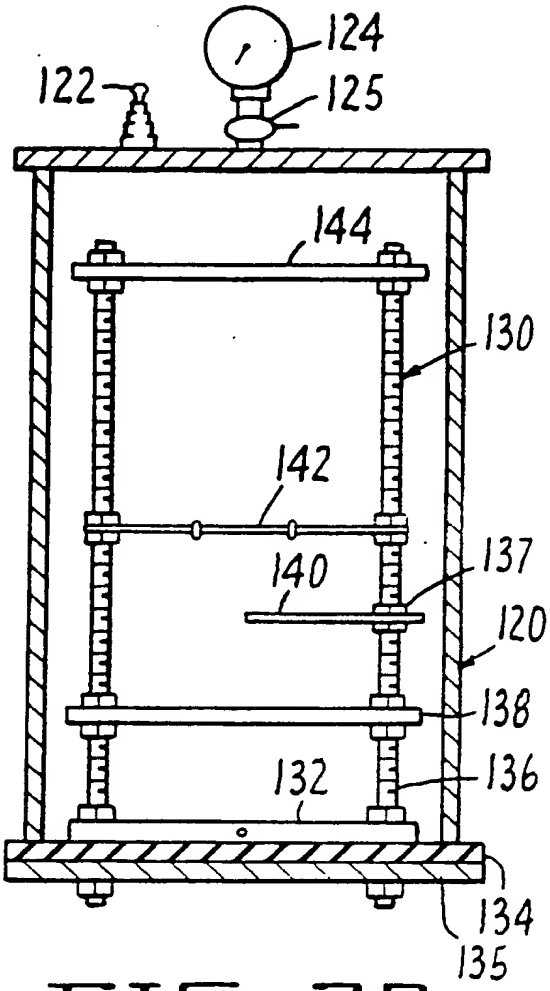

FIG.3B.

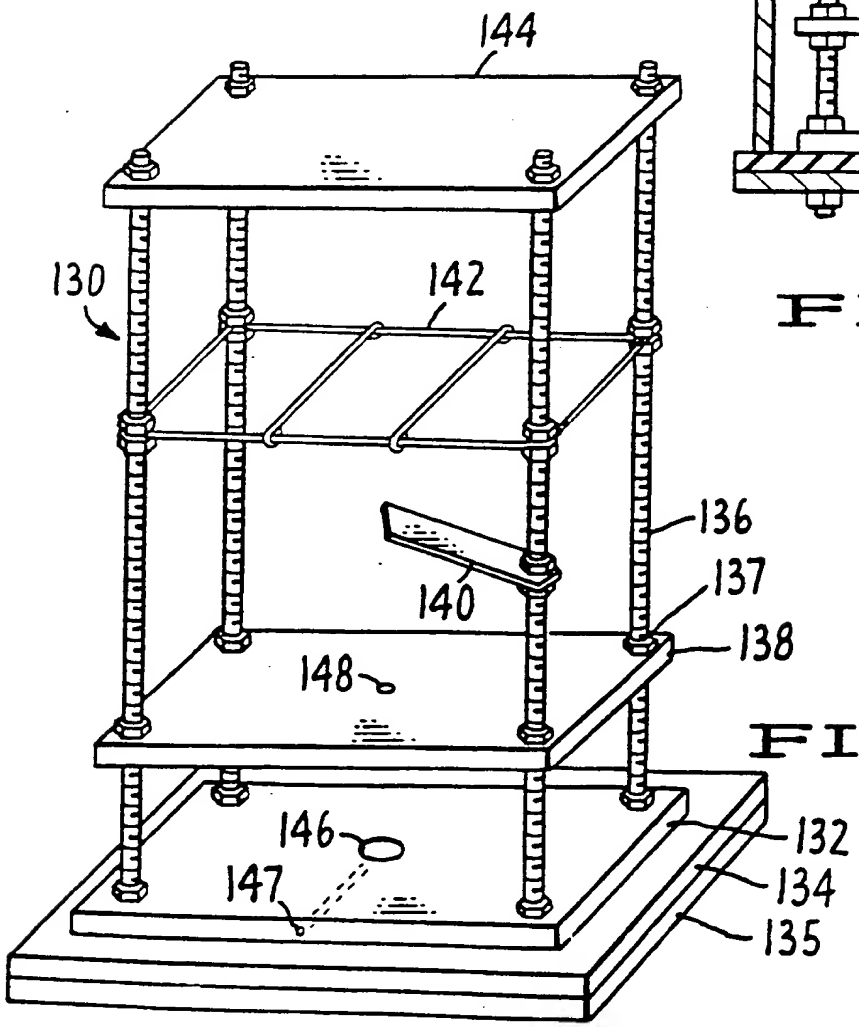

FIG.3C.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05021

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/87; F25C 3/04
U.S.Cl.: 435/172.3; 239/2.2

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	239/2.2,14.2 435/172.3 935/85

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *

USPTO Automated Patent System.
See attached sheet for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
A/Y	EP, A, 0,301,749 (CHRISTOU , et al) 01 February 1989. See entire document.	1-26 / -
A/Y	EP, A, 0,270,356 (McCABE et al) 08 June 1988. See entire document.	1-26 / -
A/Y	EP, A, 0,331,855 (SANFORD et al) 13 September 1989. See entire document.	1-26 / -, 28
A/Y, P	US, A, 4,945,050 (SANFORD et al) 31 July 1990. See entire document.	1-26 / -, 28
A/Y	Particulate Science and Technology, Volume 5, Issued 1987, Sanford et al, "Delivery of substances into cells and tissues using a particle bombardment process", pages 27-37. See entire document.	1-26 / -, 28
A	Nature, Volume 327, Issued 07 May 1987, Klein et al, "High-velocity microprojectiles for delivering nucleic acids into living cells", pages 70-73. See entire document.	1-26
	See attachment.	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 October 1991	22 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	P. Rhodes, Examiner

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Proceedings of the National Academy of Sciences USA, Volume 86, Issued October 1989, Christou et al, "Inheritance and expression of foreign genes in transgenic soybean plants", pages 7500-7504. See entire document.	1-26
Y	US, A, 4,819,878 (BAILEY et al) 11 April 1989. See entire document.	28
Y	US, A, 4,813,598 (KOSIK et al) 21 March 1989. See entire document.	28
Y	US, A, 4,573,636 (DILWORTH et al) 04 March 1986. See entire document.	28

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210,
Part II. FIELDS SEARCHED SEARCH TERMS:

SNOW MAKING, MIST